Method for Isolation and Detection of Dioctyl Phthalate from Milk Lipids

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A method has been developed to isolate dioctyl phthalate (DOP, di-(2-ethylhexyl) phthalate) from milk. Milk from an individual dairy farm was dialyzed and evaporated to dryness, and the dry residue was extracted with petroleum ether. The petroleum ether extract of dried milk was chromatographed on an alumina column as described by Hanahan. The fraction containing free fatty acids and DOP was separated by thin layer chromatography (TLC). DOP was distinguished from other phthalates by TLC and infrared spectroscopy.

Phthalic acid or its esters are used in industry in the preparation of alkyd resins, as plasticizers for various types of synthetic resins or plastics, as lubricants, and for dyes and intermediates. Short-chain alkyl esters of phthalic acid are used as insect repellents and insecticides.

The esters are fat-soluble substances and can migrate from plastic packaging into foods, particularly into fatty foods (1). DOP also migrates into maple sap from the collection tubing during collection of sap (2).

Recently Etemadi (3) reported that phthalic acid and its esters, which are frequently found in the lipid extracts of bacteria, are contaminating substances derived from the plastic containers used during the extraction of the lipids.

Nothing is mentioned in the literature about the isolation of phthalates from milk.

Current studies on milk lipids have revealed an unknown lipid component in milk from an individual dairy farm; this component has been identified as DOP by thin layer chromatography (TLC) and infrared spectrophotometry. The separation of this component from the lipids and its identification are described.

METHOD[2]

Reagents

(All solvents used were reagent grade, redistilled before use through Vigreaux-type column.)

(a) *Alumina (Al_2O_3).*—Activated, chromatographic grade (Matheson, Coleman and Bell, #9296).

(b) *Chromogenic agent.*—Iodine vapor, iodine crystals.

(c) *Solvent systems.*—(*1*) For TLC: petroleum ether-diethyl ether-acetic acid, 90:10:1 (v/v/v); chloroform-methanol-water, 65:25:4; and benzene. (*2*) For *alumina column:* $CHCl_3$-CH_3OH, 1:1 (v/v); EtOH-$CHCl_3$-H_2O, 5:2:1; and 5:2:2 (v/v/v).

(d) *Petroleum ether.*—Boiling range 30–60°C.

Apparatus

(a) *TLC equipment (plates, mounting board, drying rack, etc.).*—Obtained from DeSaga/Brinkmann Instruments Co., Inc., Great Neck, N.Y. (Glass plates.—8 × 8″, 6 × 8″, and 2 × 8″.)

(b) *Chromatographic column.*—Kontes Glass Co., E2, 400 mm long × 29.5 mm i.d., with replaceable coarse-porosity sintered glass disk and Teflon stopcock.

(c) *Freeze-dying apparatus.*—Virtis Co., Inc., or equivalent.

(d) *Dialysis tubing.* — Cellulose, containing 10% water and 25% glycerol (Arthur H. Thomas Co., Philadelphia, Pa.); free of DOP.

(e) *Oven provided with thermoregulator.*

(f) *Lamp.* — Mineralight ultraviolet lamp UVS-11 (Ultra Violet Products, Inc., San Gabriel, Calif.). (Phthalates appear as blue fluorescent spots.)

(g) *Spectrophotometer.*—Beckman IR-7, or equivalent.

TLC Technique (Qualitative) (4)

Weigh 30 g silica gel G (E. Merck & Co.) into 250 ml beaker, add 60 ml water, and stir slurry for 90 sec. Pour slurry in applicator. Pull applicator with steady motion across series of plates and let coated plates dry in position on mounting board for 20–25 min. Remove plates from mounting board, slide into drying rack, and dry at 105°C for 1 hr in forced draft oven; remove and cool.

Apply samples (8–10% solution in benzene) to plate 2 cm from bottom at 1.5 cm intervals, using 200–400 μg/spot. Develop plate in chromatographic jar containing appropriate solvent and fitted with filter paper liner to saturate atmosphere. When solvent front has risen 15 cm above line of application, remove plate and air-dry in hood to remove solvent. Place plate for few minutes in chromatographic jar filled with purple iodine vapors and iodine crystals in glass dish at bottom; yellow to brown spots indicate presence of lipids and DOP. Remove plate; mark spots or cover plate with another plate to keep iodine from evaporating off plate and spots from fading.

(*Note:* 0.27 mm silica gel G was maintained throughout the experiments, including preparative TLC.)

Extraction

Dialyze 1 L milk against 15 L distilled water, changing water 3 times daily at 2°C for 4 days to eliminate lactose and other dialyzable material. Lyophilize nondialyzable fraction by vacuum freeze-drying technique, using acetone-Dry Ice mixture to freeze samples. Extract lyophilized residue with petroleum ether by continuous stirring for 4 hr, using 1.5 L solvent/100 g milk solids. Repeat this extraction 5 more times. Filter extract and evaporate solvent with aid of stream of nitrogen.

(Milk can be also freeze-dried without dialysis and extracted as described above, but extraction is more difficult.)

(*Note:* DOP can be detected in milk lipid fraction when present in detectable amount, ca 50–60 mg DOP/1 L milk: DOP migrates slightly faster than triglycerides but slower than methyl esters of common fatty acids on TLC plates; *see* Fig. 1.)

Chromatography on Alumina Column

Prepare slurry of alumina in chloroform and pour resulting slurry into chromatographic column; prepare 29.5 × 200 mm Al_2O_3 column for 50–60 g milk fats. Dissolve fat sample in 100 ml $CHCl_3$ and apply to column. Elute column (*see* Table 1) as described by Hanahan (5) at room temperature (25°C). Use each solvent until eluate contains no residue; volumes of solvents used and fats separated are shown in Table 1. (Fats are recovered almost quantitatively (99.6%); 54 g of fats was applied to column and more than 53.8 g was recovered.)

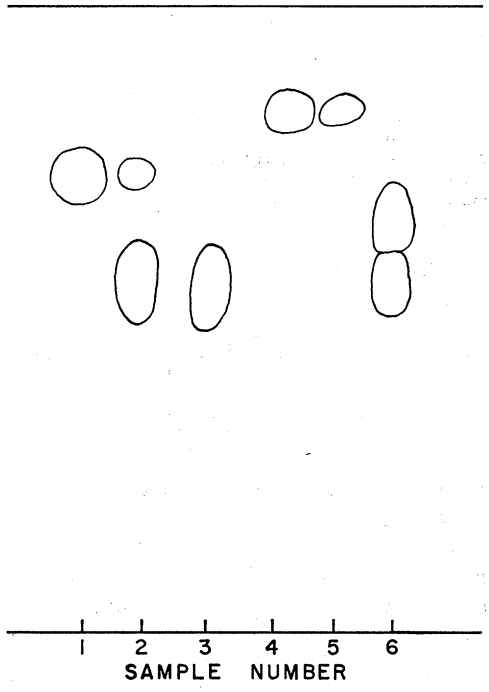


***Fig. 1*—Thin layer chromatographic diagram of phthalate-containing fraction. Solvent system: petroleum ether-diethyl ether-acetic acid (90:10:1). *1*, DOP isolated from milk; *2*, 5-2-2 fraction; *3*, oleic acid; *4*, methyl esters of 5-2-2 acids; *5*, methyl oleate; *6*, triglycerides of whole milk.**

Preparative TLC

Dissolve residue of 5:2:2 fraction (Table 1) in benzene (8–10% solution) and apply along baseline of 8 × 8″ Silica Gel G plate as streak, 2–5 mg/plate. Develop plate with petroleum ether-diethyl ether-acetic acid solvent system, *Reagents* (c), (*1*). After development, let solvent evaporate and cover 8 × 8″ plate with 6 × 8″ plate in such a manner that both sides of 8 × 8″ plate remain uncovered, and fasten tight with binder clips. Keep plates in iodine vapor, as described above, to stain open sides and delineate components. After staining, scrape off corresponding unstained DOP horizontal row with spatula into large test tube.

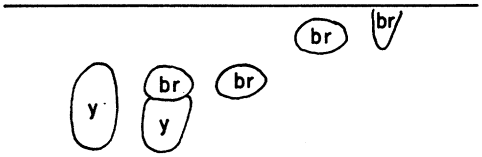
Table 1. Chromatography of milk lipids on an alumina column

Solvent	Volume of Solvent, ml	Yield, g	Lipid Components
Chloroform	2000	53.0	Glycerides, cholesterol, pigments
Chloroform-methanol (1:1)	500	traces	Lecithin, sphingomyelin
Ethanol-chloroform-water (5:2:1)	800	traces	Inositide (?)
Ethanol-chloroform-water (5:2:2)	900	0.8	Free fatty acids and DOP
Methanol	900	no residue	

Add 15 ml diethyl ether to test tube, stir with glass rod for 5–10 min, and centrifuge. Repeat extraction twice. Evaporate solvent from extract in weighed beaker by stream of nitrogen, and weigh again.

Results and Discussion

Dioctyl Phthalate (DOP).—The unknown residue was an oil. By TLC, the unknown migrated the same way as DOP (Fig. 2); also the color with iodine vapor was identical (Figs. 1–3). Infrared spectroscopy showed an absorption curve identical with that of DOP (Fig. 4).



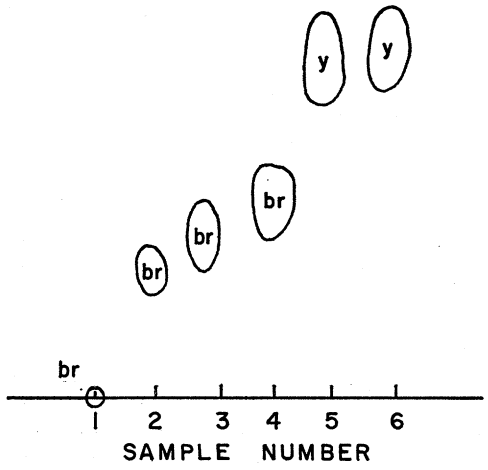


Fig. 2—**Thin layer chromatographic diagram of phthalates. Solvent system: petroleum ether-diethyl ether-acetic acid (90:10:1). br = brown; y = yellow; *1*, phthalic acid; *2*, dimethyl phthalate; *3*, diethyl phthalates; *4*, dibutyl phthalates; *5*, dioctyl phthalate (DOP); *6*, DOP isolated from milk.**

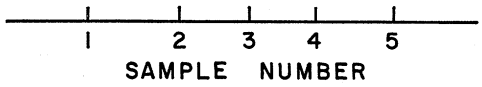


Fig. 3—**Thin layer chromatographic diagram of phthalate-containing fraction. Solvent system: chloroform-methanol-water (65:25:4). br = brown; y = yellow; *1*, DOP; *2*, 5-2-2 fraction; *3*, oleic acid; *4*, methyl oleate; *5*, whole milk triglycerides.**

Quantitative TLC showed that milk contained 80 mg DOP/L of milk in this particular case. Later samples of milk from the same farm did not contain DOP.

Infrared Spectroscopy. — Infrared spectra were obtained with a Beckman IR-7 prism-grating spectrophotometer equipped with a beam condenser. For Fig. 4A the undiluted sample was pressed thin between 25 mm salt disks. The special cell for Fig. 4B had provisions for pressing 0.05–0.3 μl droplets of liquid sample between 1.5 × 5.5 mm salt faces. The window faces projected towards the closing plane from seals in recesses of the

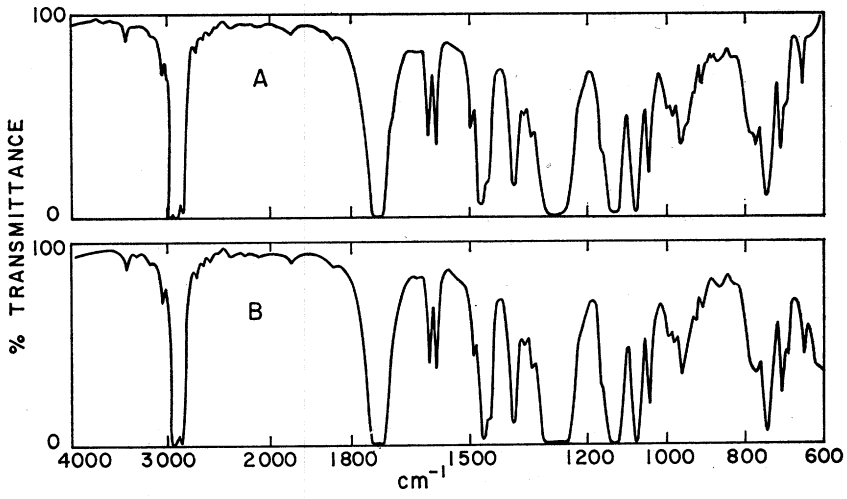


Fig. 4—A, **Authentic di-(2-ethylhexyl) phthalate (Flexol Plasticizer DOP, from Union Carbide Chemicals Co.).** *B,* **Matching microsample from milk.**

cell halves. The halves closed together by screws against pressure of a Viton O-ring in a seat spaced outside the wet zone. These conditions gave a thin wettable sample zone, entirely surrounded with receding window walls that offered no path for other capillary spread. With such arrangements, samples were pressed to uniformly occupy the transmission zone without waste. The cell construction and design (6) are expected to be further documented by public service patents from applications filed June 10, 1964. Procedures for obtaining similar spectra have been published (7, 8) and were discussed at a conference (9). The spectra of different esters of phthalic acid are generally very similar because the infrared absorption characteristics are essentially determined by the acid residue. Nevertheless, small differences can be observed, notably in the region from 800–950 cm^{-1}. The spectra of numerous phthalates were obtained and compared with the spectra of the unknown. The latter was practically identical with the spectrum of di(2-ethylhexyl)phthalate, as shown in Fig. 4.

Commercial Milk.—A sample of a commercial pooled milk was treated as described above, and no phthalate was found. However, an unknown substance was present in trace amounts in the free fatty acid fraction.

Recovery Studies.—Recovery studies were made to test the overall effectiveness and reliability of the method. DOP was added to the homogenized vitamin D milk, thoroughly stirred, and treated identically to other milk samples. The recovery of added DOP at levels of 30–40 mg/L milk was 60%. This low recovery may have been due to incomplete solution of DOP in cold milk (5°); also, the flask containing DOP-milk and dialysis tubing was not washed out with solvent after transfer of the DOP-milk for analysis. When DOP was added directly to the milk fat sample dissolved in $CHCl_3$ at a level of 30–40 mg/L milk and separated on an alumina column, the recovery of added DOP was 80%.

Extractable Matter in Laboratory Plastic Equipment and Solvents.—The presence of phthalate in milk is uncommon. According to Wandel and Tengler (1), phthalates may migrate from plastic to food. It is important to know whether laboratory materials could release phthalates or other extractable matter.

All laboratory equipment used for this investigation of milk lipids was extracted with petroleum ether and chloroform-methanol, 2:1 (v/v), and alumina was extracted with ethanol-chloroform-water, 5:2:2 (v/v). The plastic tubing and plastic bags were cut into small pieces, extracted twice with solvents by continuous stirring for 2–3 hours, and decanted. The extracts were evaporated to dry-

ness with the aid of a nitrogen stream on a steam bath, and the residues were analyzed by TLC. Reagent grade solvents (2 L each) were evaporated to dryness as described for extracts, and the residues were analyzed by TLC.

Residues of extracts from plastic tubing contained almost pure DOP. Extract from rubber tubing contained at least 20 components, and one compound had a blue fluorescence like DOP and also migrated like DOP.

Residues of solvents contained several components but none of them was DOP.

Source of DOP in Milk

This paper describes the isolation and identification of DOP from petroleum ether-soluble lipid fractions. In this particular case, milk was the research object. However, this procedure, or a slight modification, could be used to determine the phthalate in all natural lipid fractions.

The occurrence of DOP in milk is still an open question. The milk, as far as the author knows, did not come into contact with the equipment (plastic tubing) used in the laboratory.

Theoretically, the presence of DOP in milk could be explained in two ways: either DOP is secreted by the cow into the milk, or the milk is contaminated after secretion. If the DOP was secreted by the cow into the milk, it probably was in the feed. It appears most likely that the DOP entered the milk after it was secreted.

DOP has been found in petroleums (10). Phthalic acid itself and its short-chain alkyl esters have been found in lipid extracts of plant materials and microorganisms and in tobacco smoke (17 references). Diheptyl phathalate has been produced by *Alternaria kikuchiana* Tanaka fungus which produces a black spot disease in pears (11).

Phthalic acid is a relatively strong acid; when injected into man (12), rabbit (13), and dog (14) it is excreted unchanged almost quantitatively. Oral doses of esters (dimethyl, dibutyl, and di-(2-ethylhexyl)) are not completely absorbed. These esters are likely to be hydrolyzed *in vivo,* yielding phthalic acid and an alcohol. The 2-ethylhexyl ester (DOP) has been shown to be hydrolyzed in man, rats, rabbits, and dogs (15); DOP is a contaminant of soil samples (16).

Acknowledgments

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